Supporting Information for:

Direct identification of rituximab main isoforms and subunit analysis by online selective comprehensive two-dimensional liquid chromatography – mass spectrometry

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S1. Description of sLCxLC Functionality

The principles of sLCxLC function with parallel sampling and ^{2}D separation were previously described by our group.[31] However, since the previous iteration of this instrument was completely different, and more complicated, it is important to describe here how the current implementation of the instrument functions. In addition to the component-level depiction of the instrument shown in Fig. 2 of the main manuscript, a detailed diagram showing the timing of valve switching and solvent gradient development relative to the chromatographic timescale is shown below in Fig. S1. Each separation begins with Decks One and Two in their bypass positions, with positions 1 and 1' connected to either the ^{1}D or ^{2}D flow streams through the Duo Valve. These three valves stay in these positions for the first part of the separation, until a region of the ^{1}D separation that is of interest is encountered, and sampling of the ^{1}D effluent begins. In the example shown in Fig. S1 (corresponding to separation R.C in Fig. 3), sampling begins at 36.5 min by switching Deck One so that positions 2 and 2' are connected to the ^{1}D effluent. This valve stays in this position until the desired volume of ^{1}D effluent is collected in the sample loop (see below for detailed times and flow rates). After the first portion of ^{1}D effluent has been collected, Deck One switches to the next available loop, and this process is repeated until the desired number of fractions of ^{1}D effluent have been captured and stored in the loops of Deck One. In this implementation of the instrument, the maximum number of fractions that can be stored per Deck is six – Loop 1 is used for a bypass position, and Loop 8 is used as a second bypass during the flush gradients that are used to 'clean up' the system in preparation for ^{2}D analysis of the captured ^{1}D effluent fractions. In this case, while the second dimension is still idling, at the end of the first phase of sampling Deck One and the Duo valve switch at the same time. Deck One switches to position 8, and the Duo Valve switches so that Deck One is connected to the second dimension and Deck Two is connected to the first dimension. In cases where continuous sampling using both Decks is desired (e.g., as in R.A through R.E in Fig. 3), then Deck Two also switches at this time from position 1 to position 2 so that ^{1}D effluent is captured in Loop 2 of Deck Two. In this case Deck Two would continue switching at specified intervals in the same way that Deck One did, until the desired number of fractions is stored in the loops of Deck Two. Meanwhile, as soon as the Duo Valve switches so that Deck One is connected to the ^{2}D pumping system, the contents of Loop 8 of Deck One and the capillaries connecting Deck 1 and the Duo Valve are effectively injected into the ^{2}D column. To flush these paths and elute constituents of these fluids from the ^{2}D column, a solvent gradient is executed as indicated by the red trace at the bottom of Fig. S1 (see below for specific times and compositions; this step produces a piece of the ^{2}D chromatogram indicated with the 'F' designation in Fig. 4B that is omitted during data analysis because it cannot be accurately interpreted). After flushing Loop 8 of Deck One, the previously captured fractions of ^{1}D effluent in Loops 2-6 of Deck One are injected one at a time into the ^{2}D column in reverse order relative to the order in which they were captured. This avoids cross-contamination of fractions that would occur during valve switching if they were not injected in reverse order. Following each switch of Deck One to inject a new fraction of ^{1}D effluent, a solvent gradient is executed in the second dimension to produce one of the chromatograms shown, for example, in Fig. 4B. Following the injection of all ^{1}D effluent fractions from Deck One, this valve returns to its bypass position (1 and 1' connected). As shown in Fig. S1, the time required to process all of the fractions from Deck One usually takes longer than the sampling of ^{1}D effluent using the loops of Deck Two. While waiting for the processing of fractions from Deck One to finish, Deck Two remains in position 8. At the end of processing fractions from Deck One, the Duo Valve switches

back so that Deck Two is reconnected to the ^{2}D pump, and the processing of fractions captured in Deck Two can begin. As was the case with Deck One, loop 8 and the connection capillaries are again flushed and eluted from the ^{2}D column before injection of the fractions held in loops 2-6 are injected and eluted, again in reverse order.

One minor caveat about the specific experiments described here is that a limitation of the prototype version of the program controlling the switching of Decks One and Two only allowed the use of seven out of the eight loops of Deck One. Thus, in this case we were only able to analyze 11 rather than 12 fractions of ^{1}D effluent in a continuous way in a given sLCxLC separation. This is not a conceptual limitation; the programming problem has been resolved and will not be a factor in subsequent work.

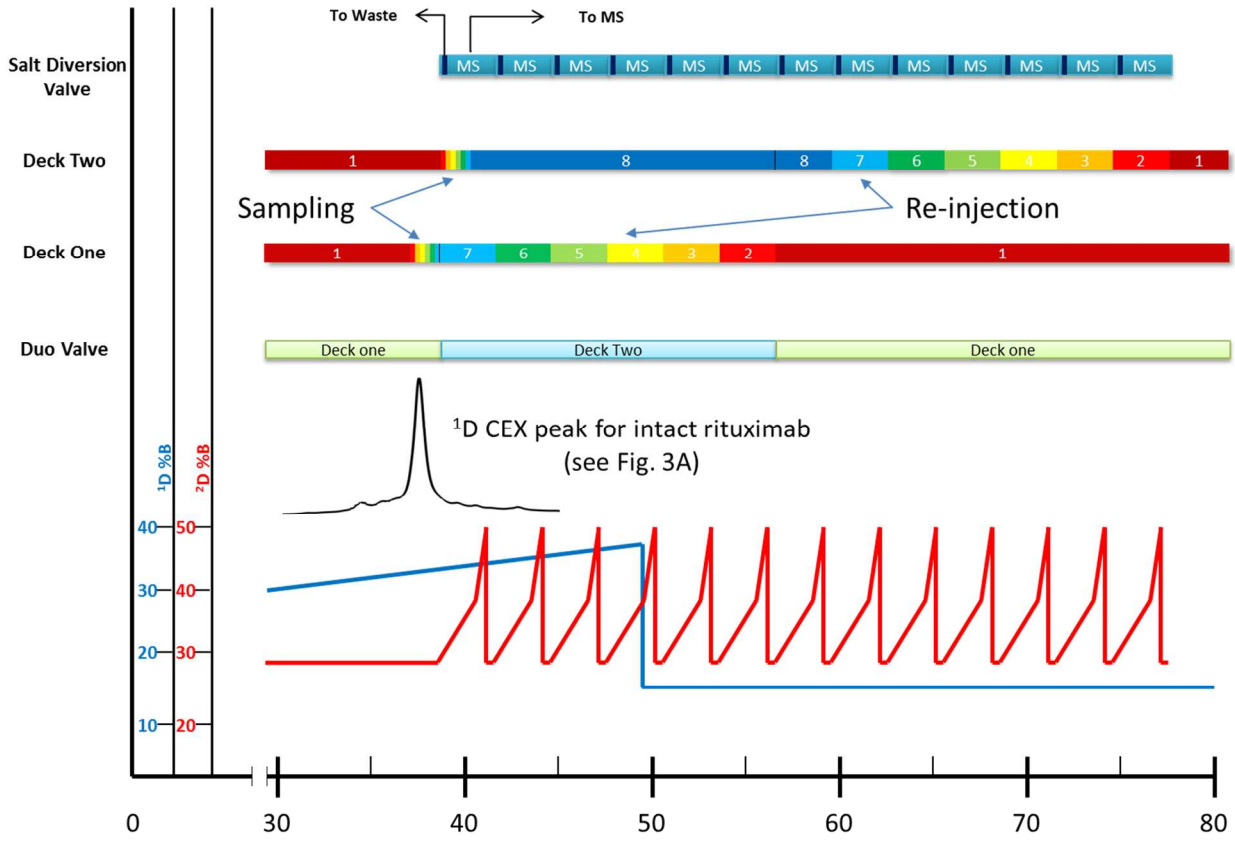

Figure S1. Detail of timing of all valve switching and solvent gradient timing. This specific diagram relates to the sLCxLC analysis of rituximab indicated as R.C in Fig. 3A.

S.2 Characterization of *IdeS*-Digested Rituximab

The composite 2D chromatogram compiled from the ^{2}D chromatograms obtained in four sLCxLC separations of the IdeS-digested rituximab sample is shown in Fig. S2B. Here again, white ellipses indicate the locations of detected protein peaks. As was the case with the intact rituximab, many peaks are observed with very similar ^{2}D retention times (about 1.58 min) even

though they have very different ^{1}D CEX retention. To make it more clear that these peaks (i.e., #1, 2, 4, 7, and 8) have distinct maxima in the first dimension and are not simply due to carryover from one fraction to the next, Fig. S2C shows a reconstructed ^{1}D chromatogram based on the signal observed in the ^{2}D detector between 1.55 and 1.62 min. This is analogous to an extracted-ion chromatogram in mass spectrometry, except that it represents a kind of 'extracted ^{2}D retention' chromatogram. We see that there are valleys between several small peaks in this chromatogram, which proves that they are real, and not due to carryover from one fraction to the next. With the exception of peak 3, all of these peaks have nominal masses of 96.7 kDa, suggesting that they are all F(ab)'$_2$ fragments that either vary slightly in composition, or present a slightly different effective charge to the CEX column. In peaks 3-6, however, we see a case where the resolving power of each ^{2}D separation is useful. Peak 3 has a mass of 48,357 Da suggesting that it is a Fab rather than a F(ab)'$_2$ fragment. Peaks 4 and 5 have masses of 96,713 Da, whereas peak 6 has a mass of 96,745 Da, which we interpret as resulting from oxidation.[9]

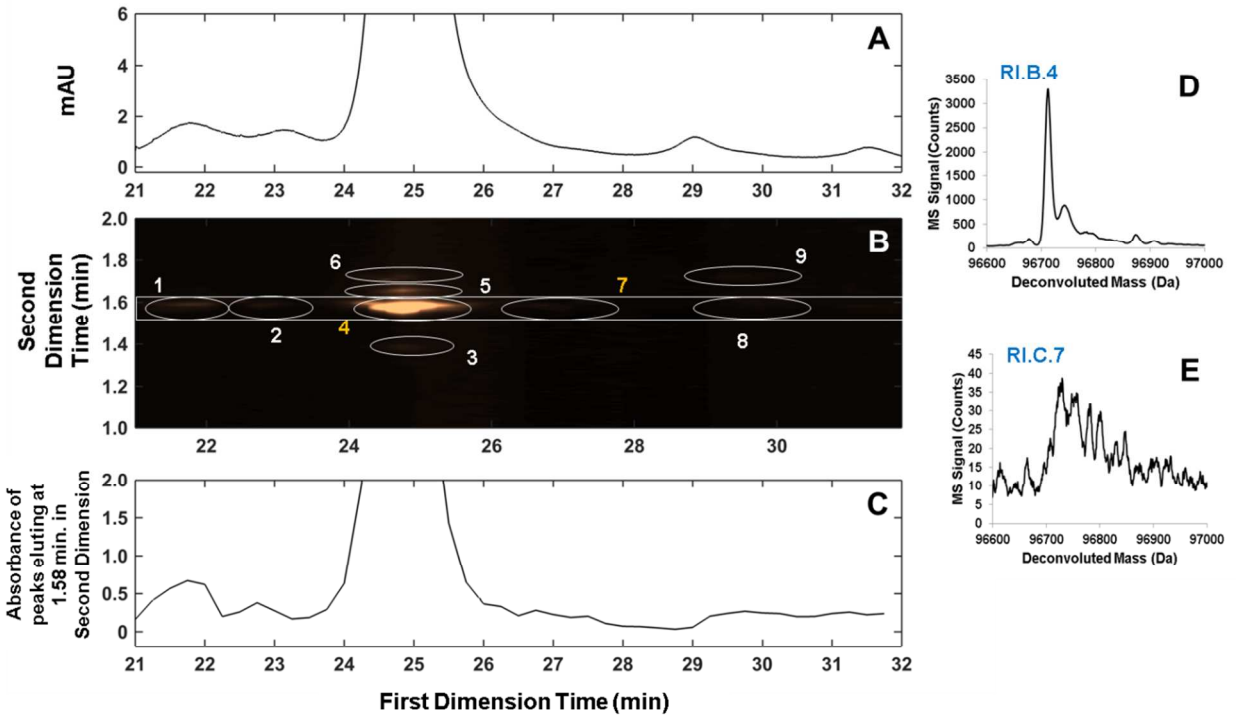

Figure S2. High resolution view of the 2D separation of F(ab)'$_2$ fragment peaks resulting from IdeS digestion of rituximab. Panel A shows the same signal shown in Fig. 3B, but magnified to emphasize the main F(ab')$_2$ fragment peak (ca. 25 min) and closely eluting minor species. Panel (B) shows the 2D chromatogram constructed from four sLCxLC analyses of the region from 21 to 32 min of the ^{1}D separation. White ellipses indicate the locations of peaks in the 2D space identified by manual inspection of the ^{2}D chromatograms. Panel (C) shows a reconstructed 1D chromatogram based on the signal from 1.55-1.62 min in each 2D chromatogram. The presence of several peaks in this reconstructed chromatogram constitutes evidence that the minor 2D peaks indicated in panel (B) are real. Signals in panels A-C are from detection by UV absorption of light at 280 nm. Panels (D) and (E) are representative deconvoluted protein mass spectra for the major F(ab)'$_2$ fragment peak and one of the minor closely eluting species.

In addition to the thorough analysis of the F(ab)'$_2$ fragments as discussed above and shown in Fig. S2, sLCxLC was also used to flexibly target and characterize the main FC fragment around 9 min. in Fig. 3B, as well as the smaller peak eluting around 13.8 min. In other words, sLCxLC was used to examine in detail the ^{1}D CEX peaks eluting in two windows that were separated too far in time to efficiently collect fractions across the entire window, and this would not have been a good use of analysis time anyway because no ^{1}D peaks are observed in the region from about 9.3 to 13 min. The resulting 2D chromatograms from each targeted section of the ^{1}D separation are shown below in Panels B and C of Fig. S3.

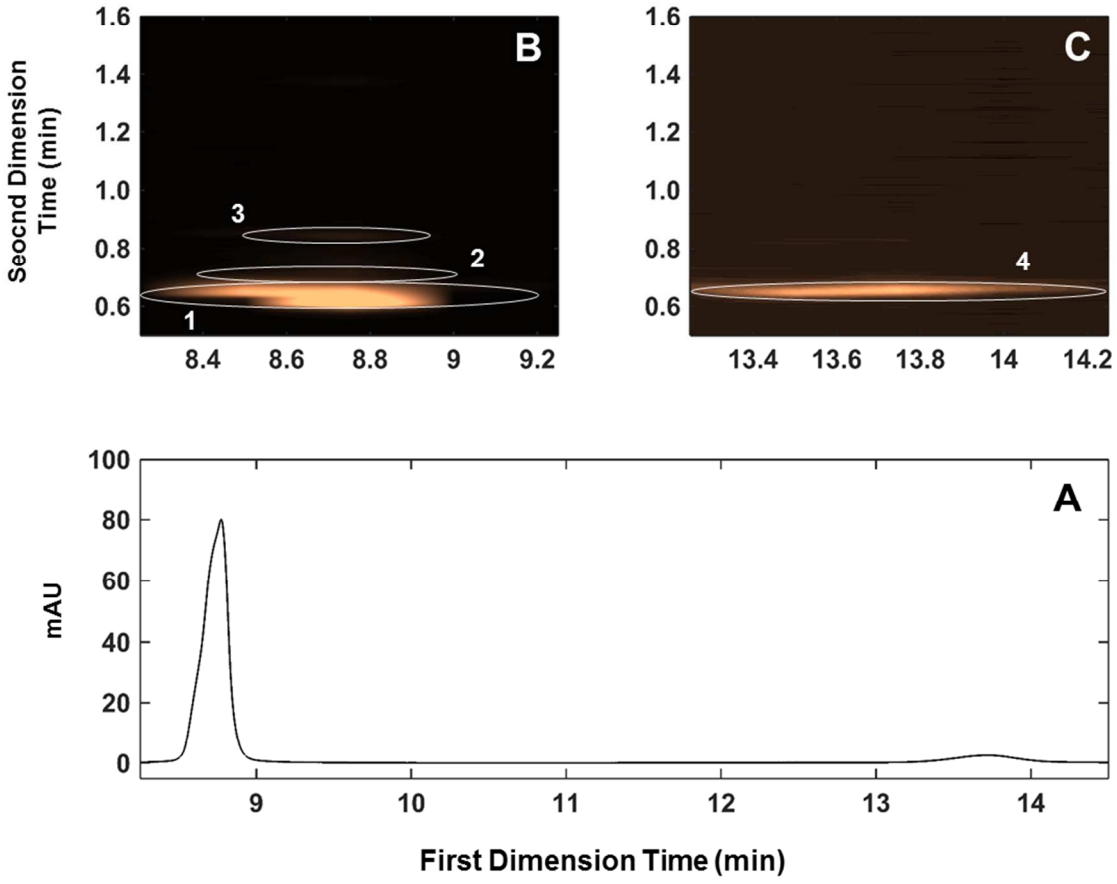

Figure S3. Flexible sLCxLC separation of rituximab digested with *IdeS*. Panel (A) shows the same ^{1}D separation shown in Fig. 3B, but magnified to emphasize the region containing the major FC fragment peak (ca. 8.75 min). Panels (B) and (C) show the 2D chromatograms resulting from targeting the ^{1}D regions from 8.25 to 9.50, and 13.25 to 14.50 min, respectively, in a single sLCxLC analysis. White ellipses indicate the locations of peaks in the 2D space identified by manual inspection of the ^{2}D chromatograms. Signals in panels A-C are from detection by UV absorption of light at 280 nm.

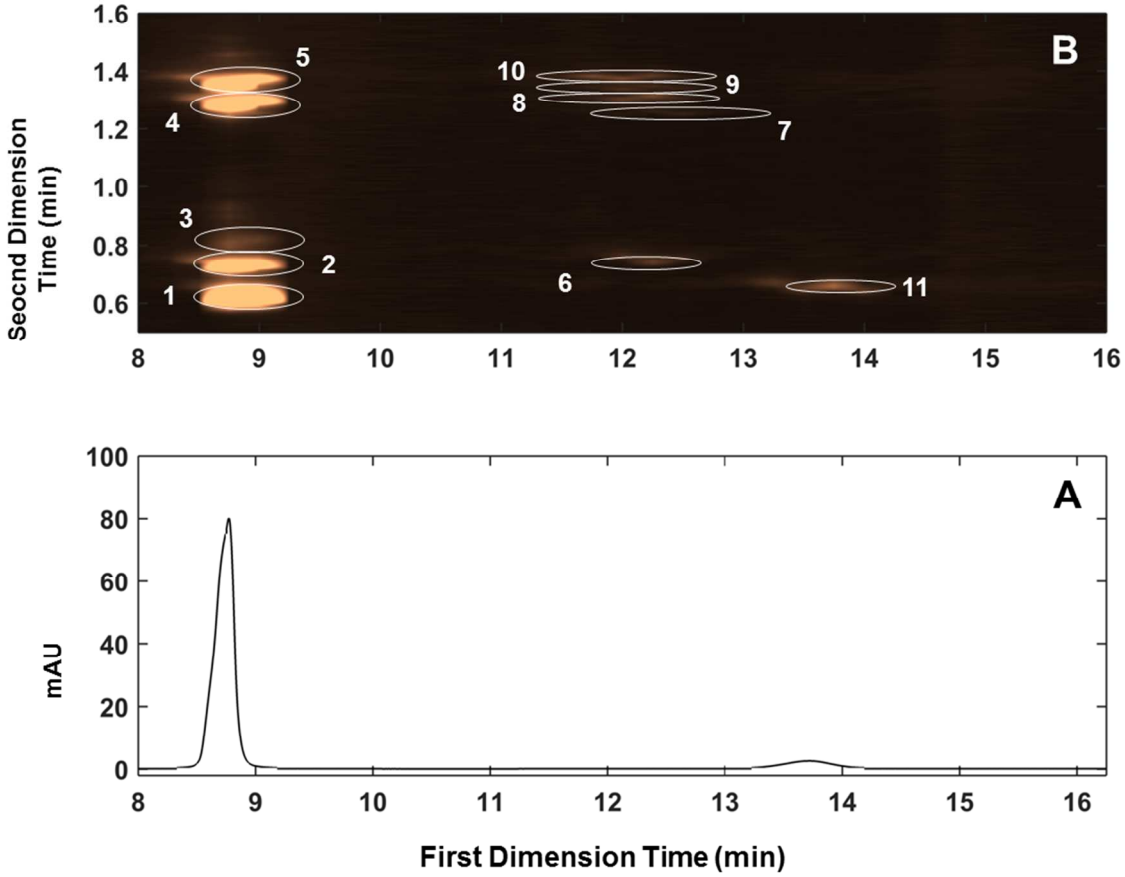

Figure S4. High resolution view of the 2D separation of fragment peaks resulting from *IdeS* digestion of rituximab, followed by reduction with DTT. Panel A shows the same signal shown in Fig. 3C, but magnified to emphasize the main peak containing the FC, LC, and Fd fragments (ca. 8.75 min) and closely eluting minor species. Panel (B) shows the 2D chromatogram constructed from three sLCxLC analyses of the region from 8.00 to 16.25 min of the ^{1}D separation. White ellipses indicate the locations of peaks in the 2D space identified by manual inspection of the ^{2}D chromatograms. Signals in panels A and B are from detection by UV absorption of light at 280 nm.

References

(1) Larson, E. D.; Groskreutz, S. R.; Harmes, D. C.; Gibbs-Hall, I.; Trudo, S. P.; Allen, R. C.; Rutan, S. C.; Stoll, D. R. *Anal. Bioanal. Chem.* **2013**, *405* (13), 4639–4653.